VOLUME 34 · NUMBER 29 · OCTOBER 10, 2016

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

HER2 Gene Amplification Testing by Fluorescent In Situ Hybridization (FISH): Comparison of the ASCO-College of American Pathologists Guidelines With FISH Scores Used for Enrollment in Breast Cancer International Research Group Clinical Trials

Michael F. Press, Guido Sauter, Marc Buyse, Hélène Fourmanoir, Emmanuel Quinaux, Denice D. Tsao-Wei, Wolfgang Eiermann, Nicholas Robert, Tadeusz Pienkowski, John Crown, Miguel Martin, Vicente Valero, John R. Mackey, Valerie Bee, Yanling Ma, Ivonne Villalobos, Anaamika Campeau, Martina Mirlacher, Mary-Ann Lindsay, and Dennis J. Slamon

Author affiliations appear at the end of this article.

Published online ahead of print at www.jco.org on August 29, 2016.

Support information appears at the end of this article.

Authors' disclosures of potential conflicts of interest are found in the article online at www.jco.org. Author contributions are found at the end of this article.

Clinical trial information: NCT00021255 and NCT00312208.

Corresponding author: Michael F. Press, MD, University of Southern California Norris Comprehensive Cancer Center, 1441 Eastlake Ave, NOR5409, Los Angeles, CA 90033; e-mail: press@usc.edu.

0732-183X/16/3429w-3518w/$20.00

DOI: 10.1200/JCO.2016.66.6693

ABSTRACT

Purpose

ASCO and the College of American Pathologists (ASCO-CAP) recently recommended further changes to the evaluation of human epidermal growth factor receptor 2 gene (*HER2*) amplification by fluorescent in situ hybridization (FISH). We retrospectively assessed the impact of these new guidelines by using annotated Breast Cancer International Research Group (BCIRG) -005, BCIRG-006, and BCIRG-007 clinical trials data for which we have detailed outcomes.

Patients and Methods

The *HER2* FISH status of BCIRG-005/006/007 patients with breast cancers was re-evaluated according to current ASCO-CAP guidelines, which designates five different groups according to *HER2* FISH ratio and average *HER2* gene copy number per tumor cell: group 1 (in situ hybridization [ISH]–positive): *HER2*-to-chromosome 17 centromere ratio $\geq$ 2.0, average *HER2* copies $\geq$ 4.0; group 2 (ISH-positive): ratio $\geq$ 2.0, copies $<$ 4.0; group 3 (ISH-positive): ratio $<$ 2.0, copies $\geq$ 6.0; group 4 (ISH-equivocal): ratio $<$ 2.0, copies $\geq$ 4.0 and $<$ 6.0; and group 5 (ISH-negative): ratio $<$ 2.0, copies $<$ 4.0. We assessed correlations with HER2 protein, clinical outcomes by disease-free survival (DFS) and overall survival (OS) and benefit from trastuzumab therapy (hazard ratio [HR]).

Results

Among 10,468 patients with breast cancers who were successfully screened for trial entry, 40.8% were in ASCO-CAP ISH group 1, 0.7% in group 2; 0.5% in group 3, 4.1% in group 4, and 53.9% in group 5. Distributions were similar in screened compared with accrued subpopulations. Among accrued patients, FISH group 1 breast cancers were strongly correlated with immunohistochemistry 3+ status ($P < .0001$), whereas groups 2, 3, 4, and 5 were not; however, groups 2, 4 and, 5 were strongly correlated with immunohistochemistry 0/1+ status (all $P < .0001$), whereas group 3 was not. Among patients accrued to BCIRG-005, group 4 was not associated with significantly worse DFS or OS compared with group 5. Among patients accrued to BCIRG-006, only group 1 showed a significant benefit from trastuzumab therapy (DFS HR, 0.71; 95% CI, 0.60 to 0.83; $P < .0001$; OS HR, 0.69; 95% CI, 0.55 to 0.85; $P = .0006$), whereas group 2 did not.

Conclusion

Our findings support the original categorizations of *HER2* by FISH status in BCIRG/Translational Research in Oncology trials.

INTRODUCTION

Amplification and overexpression of the human epidermal growth factor receptor type 2 gene (*HER2/ERBB2*) is an established therapeutic target in breast and gastric carcinomas.[1-5] Because this alteration is found in other carcinomas at varying prevalence,[6-8] the alteration may also prove therapeutically useful in some of these cancers. Although not associated with overexpression,[9] activating mutations in extracellular and tyrosine kinase domains of *HER2/ERBB2* in breast cancer respond to small-molecule inhibitors, such as lapatinib and neratinib, but to date, these findings have been restricted to preclinical model systems.[10]

As humanized anti-HER2 monoclonal antibodies[2-5,11,12] and small-molecule kinase inhibitors[13,14] of HER2 are established as effective only in cancers with amplification and overexpression, the US Food and Drug Administration (FDA) has required a companion diagnostic to select patients for these treatments. Because of reported discrepancies in HER2 testing results using HER2 companion diagnostics, ASCO and College of American Pathologists (ASCO-CAP) convened a panel to standardize performance and interpretation of these HER2 diagnostic assays.[15,16] This panel was recently reconvened, and new guidelines were once again issued for HER2 test results.[17,18] Because these recommendations differ from past ASCO-CAP and FDA recommendations—and given the fact that *HER2* status by fluorescent in situ hybridization (FISH) assay was an entry criterion for the Breast Cancer International Research Group (BCIRG)/Translational Research in Oncology (TRIO) clinical trials of trastuzumab and lapatinib in the treatment of breast and gastric cancers, respectively, in the adjuvant and advanced disease settings,[4,5,13,14,19-23]—we decided to retrospectively re-evaluate our interpretations of the *HER2* FISH assays from three BCIRG clinical trials.[4,19,24] These trials now have long-term clinical follow-up data available[4,19,25] that facilitate determination of whether the new *HER2* guidelines for FISH are clinically useful and predictive of known outcomes. In the current study, we compared the original FDA-approved criteria for *HER2* gene amplification with current ASCO-CAP guidelines, assessed the number of cases in each guidelines group, and determined whether new ASCO-CAP FISH testing criteria used to define each of the five *HER2* FISH groups are correlated with characteristics known to be associated with *HER2* gene amplification, such as HER2 protein overexpression, worse clinical outcomes (disease-free survival [DFS] and overall survival [OS]) in the absence of HER2 targeted therapy, and significant improvement in DFS and OS when such patients are treated with HER2-targeted therapy.

PATIENTS AND METHODS

Patients and Clinical Trials

Patients in BCIRG-005/006/007 trials were screened for enrollment in one of two central laboratories by using *HER2* gene amplification status determined by FISH as an enrollment criterion[4,19,21] (Fig 1). Those patients whose breast cancers were *HER2* amplified were eligible for BCIRG-006 or 007, whereas those whose breast cancers were not *HER2* amplified were eligible for BCIRG-005 (Fig 1). Criteria for amplified and not amplified that were initially used to screen for entry to these trials are summarized below and in the Data Supplement.

BCIRG-006 trial (n = 3,222) is a randomized, three-arm study of adjuvant chemotherapy with or without trastuzumab in patients with *HER2*-amplified stage I to III breast cancer who were accrued between April 2001 and March 2004.[4] Therapy in the control arm was adjuvant

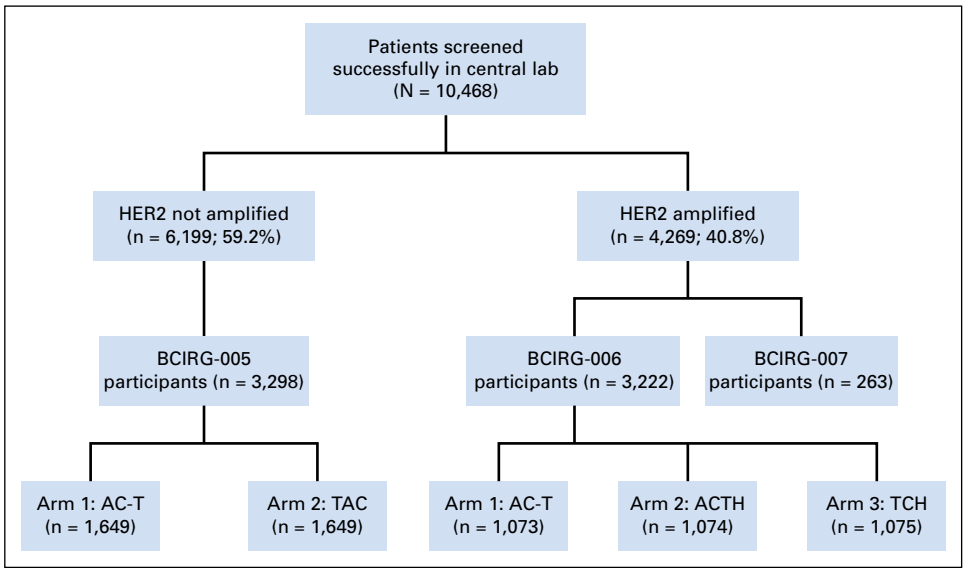

Fig 1. Specimen accountability on the basis of the CONSORT statement. Breast cancers from patients were evaluated in one of two central laboratories as either human epidermal growth factor receptor 2 gene (*HER2*) not amplified or *HER2* amplified for eligibility to one of three concurrently conducted clinical trials (BCIRG-005, BCIRG-006, AND BCIRG-007). One of the trials, BCIRG-005, required patients whose breast cancers were *HER2* not amplified and the other two trials, BCIRG-006 and BCIRG-007, required patients whose breast cancers were *HER2* gene amplified as determined with fluorescent in situ hybridization (FISH). Although 10,948 patients were screened in the Breast Cancer International Research Group central laboratories for trial accrual, complete *HER2* FISH assay results were available from 10,468 patients for a variety of reasons, including lack of invasive carcinoma in samples submitted, tissue sections that detached from slides during processing, and FISH assay failure as a result of lack of probe hybridization. AC-T, anthracycline, cyclophosphamide, and docetaxel; ACTH, anthracycline, cyclophosphamide, docetaxel, and trastuzumab; TAC, taxotere, docetaxel, and cyclophosphamide. TCH, docetaxel, carboplatin, and trastuzumab.

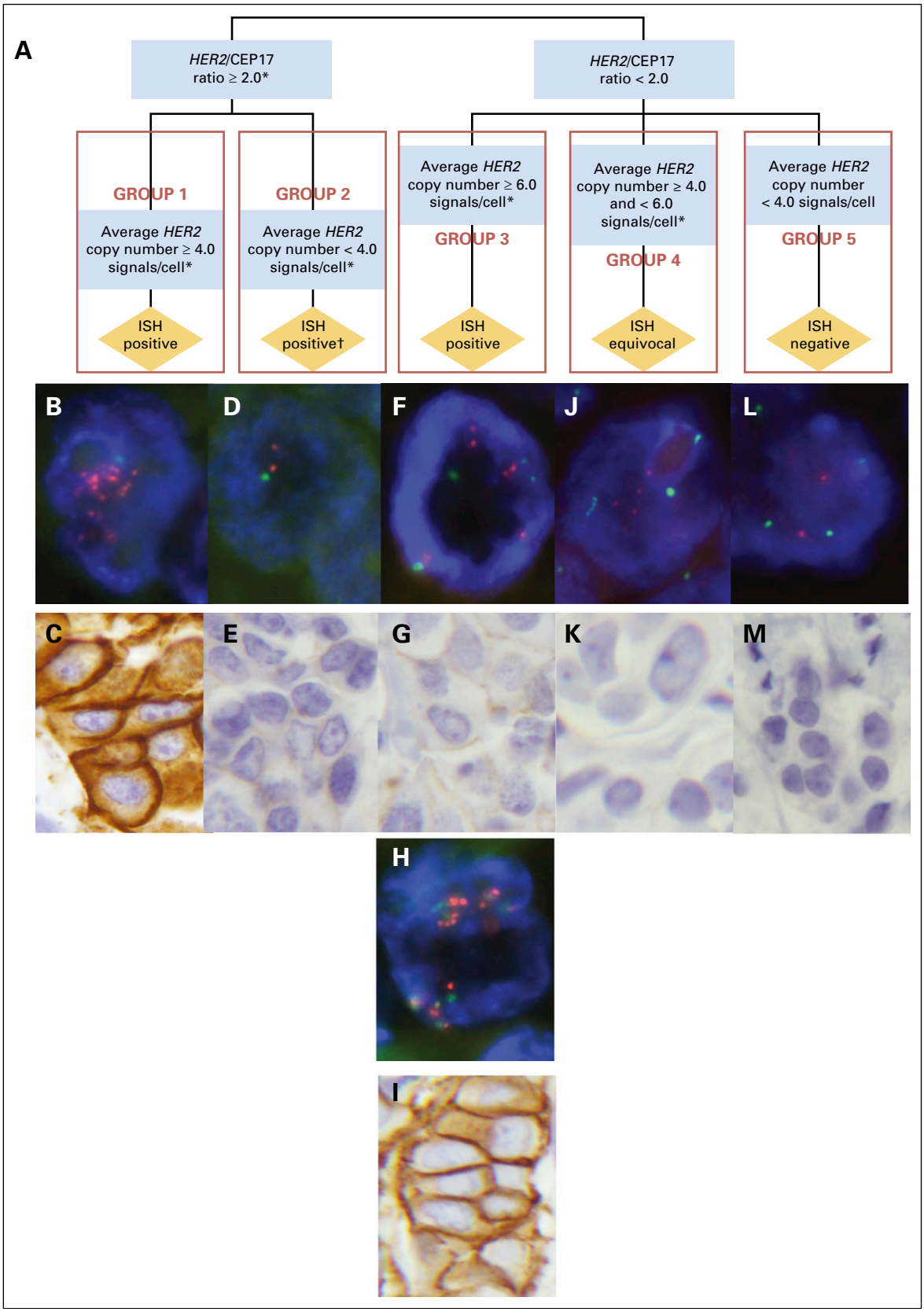

Fig 2. Schematic diagram of the ASCO and College of American Pathologists (ASCO-CAP) algorithm for human epidermal growth factor receptor 2 (HER2) testing by fluorescent in situ hybridization (FISH) as published by the ASCO-CAP guidelines committee,[17,18] modified here by introduction of the numbers 1 to 5 to identify the various ASCO-CAP FISH groups categorized, followed by FISH and immunohistochemistry (IHC) photomicrographs of representative cases from each of (continued on next page)

anthracycline, cyclophosphamide, and docetaxel (AC-T) with or without hormonal therapy depending on tumor estrogen receptor and progesterone receptor status at site investigator discretion. Therapy in the two experimental arms involved trastuzumab (H) with patients randomly assigned to either standard AC-T adjuvant chemotherapy or nonanthracycline chemotherapy with docetaxel and a platinum salt, again, with or without hormonal therapy depending on tumor estrogen receptor and progesterone receptor status. This trial demonstrated significant improvement in DFS for both trastuzumab-containing treatment arms compared with control AC-T adjuvant chemotherapy alone. Outcomes are summarized in the Data Supplement and reported elsewhere.[4,26]

BCIRG-005 clinical trial (n = 3,298) is a randomized study of concurrent (taxotere, adriamycin, and cyclophosphamide) or sequential (AC-T) adjuvant anthracycline-containing chemotherapy in patients with *HER2*-normal (nonamplified) stage II and III breast cancer who were accrued from August 2000 to February 2003. This trial demonstrated that sequential and combination regimens that incorporated three drugs were equally efficacious but differed significantly in toxicity profile. Clinical outcomes are summarized in the Data Supplement, and trial details are reported elsewhere.[19,25]

BCIRG-007 trial (n = 263), a randomized phase III trial of docetaxel and trastuzumab compared with docetaxel, carboplatin, and trastuzumab in women with *HER2*-amplified metastatic breast cancer,[24] was screened for *HER2* status by FISH concurrently with BCIRG-005 and BCIRG-006. Data for *HER2* gene amplification and expression are included in the current study; however, outcome information is not included as this trial had no control, nontrastuzumab treatment arm (Data Supplement).

Laboratory Methods

HER2 gene amplification status was determined by using FISH as described in the Data Supplement. Patients whose breast cancers were *HER2* amplified—*HER2*-to-chromosome 17 centromere (CEP17) FISH ratio ≥ 2.0—without regard to the average *HER2* gene copy number as approved by the FDA met an eligibility criterion for BCIRG-006 and BCIRG-007, whereas those whose breast cancers were *HER2* nonamplified by FDA-approved criteria met the eligibility criterion for BCIRG-005 (Fig 1). HER2 protein expression was evaluated in a blinded fashion by using the HercepTest (DAKO, Carpinteria, CA) immunohistochemical (IHC) assay (Data Supplement); however, only FISH was used for enrollment.

Breast cancers screened for enrollment into these BCIRG/TRIO trials were simultaneously screened for all three clinical trials: BCIRG-005, BCIRG-006, and BCIRG-007. As personnel in central laboratories had no knowledge of which cases were potential participants for any of the studies, all screened cases were handled in the same fashion without any distinction related to trial design. As previously described,[21] only 5% of these specimens had prior assessment for *HER2* status by FISH in local laboratories, whereas approximately 60% had been previously assessed by some HER2 IHC assay. Because of a relatively high false-positive rate (22%) among outside IHC3+ cases, outside IHC assays were not considered sufficiently accurate for accrual to or exclusion from any of the trials.[21] For current comparisons of FISH to IHC, these cases were all analyzed in the same fashion as they were initially processed, that is, without reference to their potential to be included in any particular trial. We consider this the most appropriate way to avoid introducing bias into the comparison of *HER2* gene amplification by FISH with HER2 protein expression by IHC.

(continued) the five groups. (A) Breast cancers with *HER2*-to-chromosome 17 centromere (CEP17) ratios ≥ 2.0 are divided in two groups, one with an average *HER2* gene copy number per tumor cell ≥ 4.0 (in situ hybridization [ISH] positive; our group 1) and one with an average *HER2* gene copy number per tumor cell < 4.0 (ISH positive; our group 2). Breast cancers with *HER2*-to-CEP17 ratios < 2.0 are separated into three additional groups: one with average *HER2* gene copy number per tumor cell ≥ 6.0 (ISH positive; our group 3), another with average *HER2* gene copy number per tumor cell ≥ 4.0 but < 6.0 (ISH equivocal; our group 4), and one with breast cancers that contained an average *HER2* gene copy number per tumor cell < 4.0 (ISH negative; our group 5). Therefore, according to the ASCO-CAP guidelines[17,18] breast cancers in groups 1, 2, and 3 are interpreted as ISH positive, group 4 as ISH equivocal, and group 5 as ISH negative. (B-M) ASCO-CAP guidelines algorithm ISH groups compared with observed *HER2* gene amplification status by FISH and HER2 protein expression status by IHC staining using the DAKO HercepTest IHC assay. ASCO-CAP guidelines algorithm identification of subdivisions by *HER2* FISH ratios and average *HER2* gene copy number into group 1 is categorized as ISH positive, with results as illustrated in panels B (FISH) and C (IHC); group 2 is also categorized as ISH positive, but with our contradictory results as illustrated in panels D (FISH) and E (IHC); group 3 is categorized as ISH positive, but with mixed results as illustrated in panels F (FISH), G (IHC), H (FISH), and I (IHC); group 4 is categorized as ISH equivocal, but with contradictory results as illustrated in panels J (FISH) and K (IHC); and group 5 is categorized as ISH negative, with confirmatory results as illustrated in panels L (FISH) and M (IHC). (B) ASCO-CAP group 1 breast cancer with *HER2* gene amplification by FISH, consistent with the ASCO-CAP guidelines designation of ISH positive (and Breast Cancer International Research Group [BCIRG] designation of *HER2* amplified). Average *HER2* gene copy number for this case was 16.85 copies per tumor cell, and the CEP17 copy number per cell was 2.28 with a *HER2*-to-CEP17 FISH ratio of 7.38. *HER2* signals are sufficiently numerous and are not captured in a single plain of focus in this photomicrograph so that some appear out of focus. Computer enhancement was not used for any image (BCIRG01661, original photomicrograph at 1,000×). (C) ASCO-CAP group 1 breast cancer case with HER2 protein overexpression, IHC3+ by the HercepTest IHC assay (BCIRG01661, original magnification, ×400). (D) ASCO-CAP group 2 breast cancer. Average *HER2* gene copy number for this breast cancer was 3.75 copies per tumor cell, with a CEP17 copy number of 1.80 per cell and a *HER2*-to-CEP17 FISH ratio of 2.08. This breast cancer was evaluated in the BCIRG/Translational Research in Oncology (TRIO) central laboratory as *HER2* not amplified by FISH, which contradicted the ASCO-CAP guidelines designation of ISH positive, and the patient was accrued to the BCIRG-005 trial. Of 52 patients whose breast cancers were in this group, three were accrued to BCIRG-005 and 46 were accrued to BCIRG-006 (BCIRG02899, original magnification, ×1,000). (E) ASCO-CAP group 2 breast cancer, corresponding to the breast cancer in panel D, with HER2 protein expression determined as IHC0 with HercepTest IHC assay, which contradicted the ASCO-CAP guidelines designation of ISH positive (BCIRG02899, original magnification, ×400). (F) ASCO-CAP group 3 breast cancer. One of our group 3N cases was reported to have a lack of *HER2* gene amplification by FISH in the BCIRG/TRIO central laboratory, contrary to the current ASCO-CAP guidelines designation of ISH positive. Average *HER2* gene copy number for this breast cancer was 7.35 copies per tumor cell, average CEP17 copy number was 4.20 per cell, and, therefore, there was a *HER2*-to-CEP17 FISH ratio of 1.75 (BCIRG04086, original magnification, ×1,000). (G) ASCO-CAP group 3 breast cancer. Our Group3N, with low HER2 protein expression by IHC (IHC0/1+), reported previously as *HER2* not amplified, contrary to the current ASCO-CAP guidelines designation of ISH positive (BCIRG04086, original magnification, ×400). (H) ASCO-CAP group 3 breast cancer, one of the BCIRG group 3A cases, with an average *HER2* gene copy number of 27.50 per tumor cell, an average CEP17 copy number of 20.67 per tumor cell, and, therefore, a *HER2* FISH ratio of only 1.33. Please note that the *HER2* gene signals (orange) and CEP17 signals (green) are aggregated together in a limited geographic area of the nucleus, making assessment of individual signals challenging without the aid of single band-pass filters (Data Supplement Figure S1). This breast cancer was reported as *HER2* amplified in the BCIRG/TRIO central laboratory, and the patient was accrued to BCIRG-006. This case is consistent with the ASCO-CAP guidelines designation of ISH positive (BCIRG00575, original magnification, ×1,000). (I) ASCO-CAP group 3 breast cancer, the same group3A in panel H, with HER2 protein overexpression by IHC (IHC3+ by HercepTest), consistent with the ASCO-CAP guidelines designation of ISH positive (BCIRG00575, original magnification, ×400; Data Supplement Figure S1E). (J) ASCO-CAP group 4 breast cancer, referred to by the current ASCO-CAP guidelines as ISH equivocal. BCIRG/TRIO central laboratory reported the case as *HER2* not amplified by FISH, with an average *HER2* gene copy number of 4.22 per tumor cell, an average CEP17 copy number of 2.23 per tumor cell, and, therefore, an *HER2*-to-CEP17 FISH ratio of 1.89. The patient was randomly assigned to BCIRG-005 (BCIRG01911, original magnification, ×1,000). (K) ASCO-CAP group 4 breast cancer, as in panel J, with low HER2 protein expression by HercepTest (IHC0; BCIRG01911, original magnification, ×400). (L) ASCO-CAP group 5 breast cancer, consistent with the guidelines designation of ISH negative, which was reported by the BCIRG/TRIO central laboratory as *HER2* not amplified by FISH. The case had an average *HER2* gene copy number of 1.35 per tumor cell, with 1.50 CEP17 copies per cell and an *HER2*-to-CEP17 ratio of 0.90 (BCIRG04095, original magnification, ×1,000). (M) ASCO-CAP group 5 breast cancer, see panel L, with low HER2 protein expression by IHC with HercepTest (IHC0), consistent the ASCO-CAP guidelines designation of ISH negative (BCIRG04095, original magnification, ×400). This figure has been modified with permission from Figure 3 of the previously published article by Wolff et al.[17] Copyright 2013 American Society of Clinical Oncology.

Interpretation of FISH Assays According to ASCO-CAP Guidelines

We re-evaluated HER2 status of all samples for the current study by using FISH according to the new ASCO-CAP guidelines, which separates in situ hybridization (ISH) into five groups (Fig 2). Three of these groups identify breast cancers that are ISH positive, one ISH equivocal, and one ISH negative. Breast cancers with *HER2*-to-CEP17 ratios of ≥ 2.0 are divided in two groups, one with an average *HER2* gene copy number of ≥ 4.0/tumor cell (our group 1) and one with an average *HER2* gene copy number of < 4.0/tumor cell (our group 2). Breast cancers with *HER2*-to-CEP17 ratios of < 2.0 are divided into three additional groups: one with average *HER2* gene copy number of ≥ 6.0/tumor cell (our group 3), which is also classified as ISH positive; another with average *HER2* gene copy number of ≥ 4.0 but < 6.0/tumor cell (our group 4), which is classified as ISH equivocal; and one with breast cancers that contain an average *HER2* gene copy number of < 4.0/tumor cell (our group 5), which is classified as ISH negative. According to the newly proposed ASCO-CAP guidelines[17,18] breast cancers in groups 1, 2, and 3 are interpreted as ISH positive, group 4 as ISH equivocal, and group 5 as ISH negative (Fig 2).

Statistical Methods

Standard statistical methods (Data Supplement) were used to assess significance for associations between ASCO-CAP FISH groups and HER2 protein expression (Friedman tests and χ^2 tests) and clinical outcomes (log-rank tests) in BCIRG-005[19,25] and BCIRG-006.[4,26] Hazard ratios (HRs) were estimated by using Cox proportional hazards regression models.

RESULTS

To determine what proportion of breast cancers are in each ASCO-CAP category, we re-examined our *HER2* FISH assessments from the BCIRG clinical trials conducted from 2000 to 2004—BCIRG-005, BCIRG-006, and BCIRG-007—and reclassified all screened cases into five groups according to the new ASCO-CAP guidelines (Table 1 and Fig 2).

The distribution by ASCO-CAP ISH group among the 10,468 patients whose breast cancers were successfully screened for enrollment into the three BCIRG/TRIO trials demonstrates that 40.8% were in group 1, 0.7% in group 2, 0.5% in group 3, 4.1% in group 4, and 53.9% in group 5 (Table 1 and Fig 3). A similar distribution was observed among randomly assigned patients whose cancers had FISH assay results available for analysis (Table 1) as well as those randomly assigned whose breast cancers were also evaluated by the HercepTest for HER2 protein expression (Table 1).

As expected, there was a significant association between increasing *HER2* FISH ratios and increasing IHC scores among those breast cancers for which both an *HER2* FISH assessment and an HER2 protein expression assessment by HercepTest IHC assay were available ($P < .0001$; Table 2). Similarly, an association was also observed between increasing average *HER2* gene copy number per tumor cell and increasing IHC scores ($P < .0001$; Table 2). Assessment of *HER2* gene amplification status typically involves an evaluation of both average *HER2* gene copy number per tumor cell and *HER2*-to-CEP17 ratio. The new ASCO-CAP guidelines have formalized this evaluation to create five different groups (Table 1 and Fig 2), which we evaluated by group to determine if HER2 protein—either low expression or overexpression—is associated with each ASCO-CAP FISH group (Table 2).

Table 1. *HER2* FISH Assay Results From BCIRG Clinical Trials According to ASCO-CAP Guidelines Categories

ASCO-CAP FISH Group	Description of HER2 FISH Category	No. of Cases (%)
HER2 FISH Groups of Breast Cancers Screened for Patient Enrollment Onto BCIRG Trials, 2000-2004		
1	Ratio ≥ 2.0, *HER2* average ≥ 4.0	4,269 (40.8)
2	Ratio ≥ 2.0, *HER2* average < 4.0	71 (0.7)
3	Ratio < 2.0, *HER2* average ≥ 6.0	55 (0.5)
4	Ratio < 2.0, *HER2* average ≥ 4.0, < 6.0	432 (4.1)
5	Ratio < 2.0, *HER2* average < 4.0	5,641 (53.9)
Total*		10,468* (100.0)
HER2 FISH Assay Groups for Patients Randomly Assigned to a BCIRG Trial		
1	Ratio ≥ 2.0, *HER2* average ≥ 4.0	3,321 (49.9)
2	Ratio ≥ 2.0, *HER2* average < 4.0	52 (0.8)
3	Ratio < 2.0, *HER2* average ≥ 6.0	16 (0.2)
4	Ratio < 2.0, *HER2* average ≥ 4.0, < 6.0	183 (2.8)
5	Ratio < 2.0, *HER2* average < 4.0	3,079 (46.3)
Total		6,651†
HER2 FISH Assay Groups Among Patients Randomly Assigned to a Trial and With HER2 IHC‡ Assay Results Available		
1	Ratio ≥ 2.0, *HER2* average ≥ 4.0	2,040 (47.1)
2	Ratio ≥ 2.0, *HER2* average < 4.0	35 (0.8)
3	Ratio < 2.0, *HER2* average ≥ 6.0	9§ (0.2)
4	Ratio < 2.0, *HER2* average ≥ 4.0, < 6.0	134 (3.1)
5	Ratio < 2.0, *HER2* average < 4.0	2,113 (48.8)
Total		4,331 (100)

Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

*Although 10,948 patients were screened in the BCIRG central laboratories for trial accrual, complete *HER2* FISH assay results were not available from 480 cases for a variety of reasons, including lack of invasive carcinoma in samples submitted, tissue sections that detached from slides during processing, and FISH assay failure as a result of lack of probe hybridization.

†Although 3,298 patients enrolled in BCIRG-005, 3,222 enrolled in BCIRG-006, and 263 enrolled in BCIRG-007 study for a total of 6,783 patients, data were available for 6,676, with 24 missing either average *HER2* copy number or the ratio, and one randomly assigned patient did not enroll.

‡HER2 IHC assay results using the HercepTest.

§The Data Supplement shows HER2 IHC assay results for 25 cases, with results of the laboratory-developed 10H8-IHC assay,[21,37,39] instead of the HercepTest.

HER2 Protein Expression by IHC in Each ASCO-CAP FISH Group

We determined whether *HER2* ISH-positive breast cancers, categorized by the new ASCO-CAP guidelines as groups 1, 2, and 3, were correlated with HER2 protein overexpression or, alternatively, low expression. As described in the Data Supplement, we found that only breast cancers in group 1 (FISH ratio ≥ 2.0, average *HER2* copy number/cell ≥ 4.0) were significantly associated with HER2 overexpression (IHC3+), with 75% of these showing either IHC2+ (28%) or IHC3+ (47.3%) immunostaining ($P < .0001$; Table 2).

In contrast, breast cancers from group 2 (FISH ratio ≥ 2.0, average *HER2* copy number/cell < 4.0) were associated with low HER2 expression, not overexpression ($P = .007$), as > 90% showed either IHC0 or IHC1+ immunostaining (Table 2), whereas breast cancers in group 3 (FISH ratio < 2.0, average *HER2* copy number/cell ≥ 6.0) were not significantly ($P = .3881$) associated with either

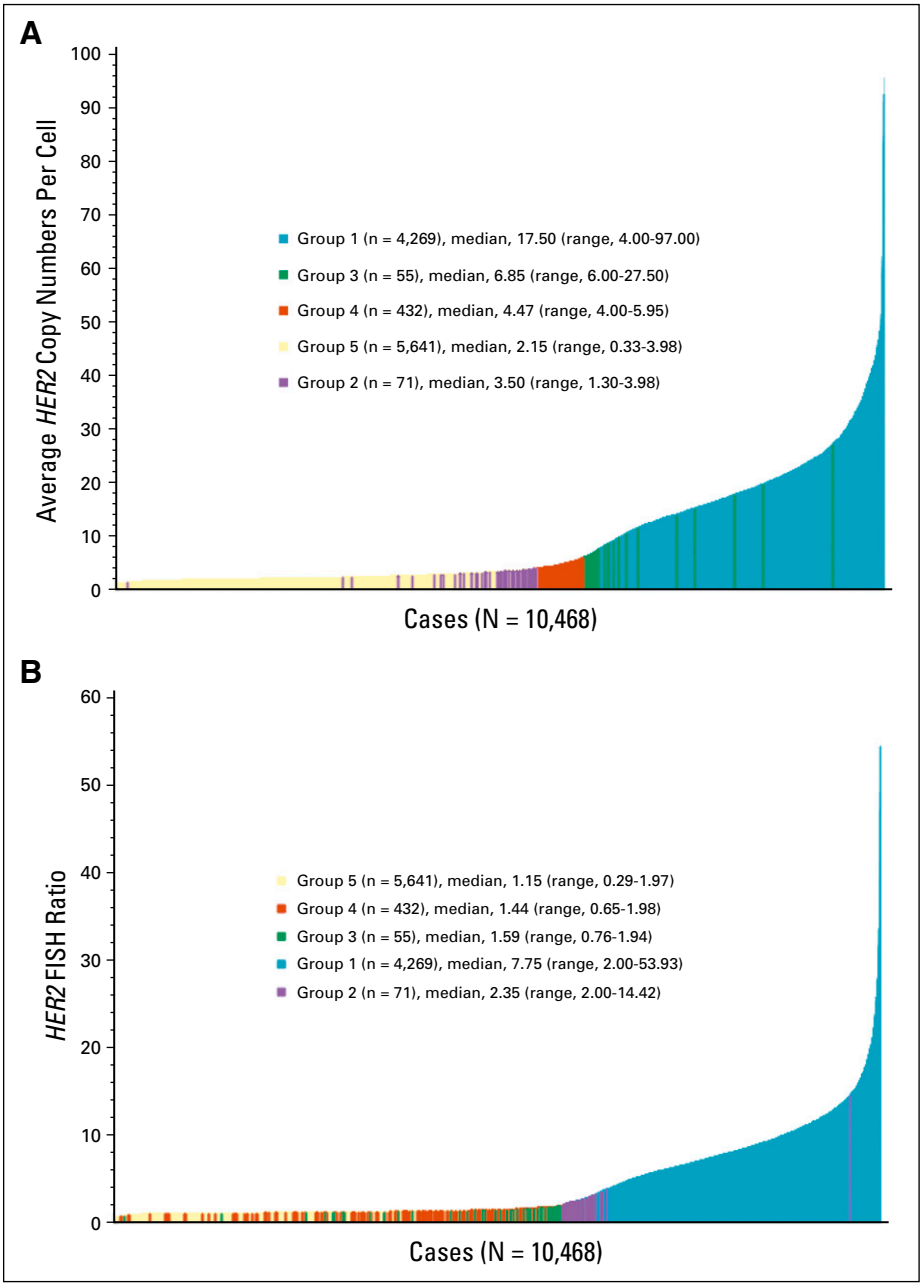

Fig 3. Distribution of average human epidermal growth factor receptor 2 gene (*HER2*) copy number and *HER2* FISH ratios among breast cancers successfully screened for enrollment into Breast Cancer International Research Group trials from 2000 to 2004. (A) Plot of average *HER2* gene copy number per tumor cell nucleus from lowest to highest, with cases identified according to the ASCO and College of American Pathologists (ASCO-CAP) guidelines as groups 1 (blue), 2 (purple), 3 (green), 4 (orange), and 5 (yellow; N = 10,468. (B) Plot of *HER2* FISH ratios from lowest to highest, as in panel A, with identification of ASCO-CAP groups 1 (blue), 2 (purple), 3 (green), 4 (red), and 5 (yellow; N = 10,468).

overexpression or low expression. Breast cancers in ASCO-CAP ISH groups 4 and 5—ISH equivocal and ISH negative, respectively—were also significantly associated with low HER2 expression (both $P < .0001$; Table 2).

Breast cancers of group 3 (FISH ratio < 2.0, average *HER2* copy number/cell ≥ 6.0) were composed of two different groups of breast cancers, a substantial majority (76%) of which were associated with low HER2 expression, whereas a minority (Data Supplement, Table S1 and Fig S2) showed HER2 overexpression.

Clinical Outcomes by ASCO-CAP ISH Groups

Because *HER2* amplification is a known adverse prognostic marker for shorter DFS and OS and predictive of improved outcomes with trastuzumab therapy, we used these outcomes to determine whether ASCO-CAP FISH groups were associated with particular end points, as expected for either HER2-positive disease or HER2-negative disease. Because the natural history of *HER2* gene amplification and overexpression in patients with breast cancer is associated with worse DFS and OS in the absence of HER2-targeted therapy[8,27,28] and with significantly improved DFS and OS with HER2-targeted therapy,[2-5,20,29,30] we have used these clinical outcomes to support the assignment of the various FISH groups as either amplified or not amplified as summarized below.

ASCO-CAP group 1 (ISH positive), HER2-to-CEP17 ratio $\geq$ 2.0 and average HER2 copy number $\geq$ 4.0 per tumor cell. As expected, those patients whose breast cancers were *HER2* amplified, with *HER2* FISH ratios of ≥ 2.0 and average *HER2* copy

Table 2. Comparison of *HER2* FISH Ratios and Average *HER2* Gene Copy Numbers With HER2 Protein Expression by HercepTest IHC Scores in BCIRG Clinical Trials

Overall Comparison of *HER2* FISH Ratios and *HER2* Copy Numbers With HER2 Protein by HercepTest IHC Scores								
PathVysion *HER2* FISH Assay		HercepTest IHC Score*						
HER2 FISH Ratio	Average *HER2* Copy Number per Cell	0	1+	2+	3+	Total	*P*†	ASCO-CAP FISH Group
< 2.0	—	2,098 (93.0%)	137 (6.1%)	19 (0.8%)	3 (0.1%)	2,257 (100%)	< .0001†	NA
2.00-5.0	—	170 (35.4%)	95 (19.8%)	104 (21.6%)	111 (23.1%)	480 (100%)		NA
5.01-10.0	—	64 (6.7%)	112 (11.7%)	288 (30.1%)	493 (51.5%)	957 (100%)		NA
> 10.0	—	30 (4.7%)	65 (10.2%)	181 (28.4%)	361 (56.7%)	637 (100%)		NA
Total‡	—	2,362	409	592	968	4,331‡		
—	< 4.0	2,017 (93.6%)	122 (5.7%)	14 (0.6%)	1 (0.05%)	2,154 (100%)	< .0001†	NA
—	4.01-6.0	166 (72.2%)	44 (19.1%)	17 (7.4%)	3 (1.3%)	230 (100%)		NA
—	6.01-8.0	48 (47.5%)	23 (22.8%)	19 (18.8%)	11 (10.9%)	101 (100%)		NA
—	8.01-10.0	25 (20.3%)	27 (22.0%)	34 (27.6%)	37 (30.1%)	123 (100%)		NA
—	> 10.0	107 (6.2%)	193 (11.2%)	510 (29.5%)	916 (53.1%)	1,726 (100%)		NA
Total‡		2,363	409	594	968	4,334‡		
Comparison of *HER2* FISH Ratios and Copy Numbers With HER2 Protein by HercepTest Scores According to ASCO-CAP Groupings								
< 2.0	< 4.0	1,988 (94.1%)	114 (5.4%)	10 (0.5%)	1 (0.05%)	2,113 (100%)	< .0001§	Group 5
	≥ 4.0-5.99	105 (78.4%)	21 (15.7%)	7 (5.2%)	1 (0.7%)	134 (100%)	< .0001§	Group 4
	≥ 6.0	5 (55.6%)	2 (22.2%)	1 (11.1%)	1 (11.1%)	9 (100%)	.3881§	Group 3
	Total	2,098 (93.0%)	137 (6.1%)	18 (0.8%)	3 (0.1%)	2,256 (100%)	< .0001§	Groups 3-5
≥ 2.0	< 4.0	24 (68.6%)	8 (22.9%)	3 (8.6%)	0 (0%)	35 (100%)	< .0007‖	Group 2
	≥ 4.0-5.99	65 (65.7%)	22 (22.2%)	10 (10.1%)	2 (2.0%)	99 (100%)	< .0001‖	Group 1
	≥ 6.0	175 (9.0%)	242 (12.5%)	561 (28.9%)	963 (49.6%)	1,941 (100%)	< .0001¶	Group 1
	Total	264 (12.7%)	272 (13.1%)	574 (27.7%)	965 (46.5%)	2,075 (100%)	< .0001#	
Total		2,362	409	592	968	4,331		

NOTE. Data from the BCIRG-007 trial comparing FISH with IHC are included in Table 2 but not in Table 3 of outcomes, because BCIRG-007 lacks a nontrastuzumab control arm.

Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; NA, not applicable.

*HercepTest scores were not available for 2,336 cases.

†*P* value of Friedman test for increasing FISH ratio with increasing IHC.

‡The *HER2* FISH ratio was not available for three cases.

§*P* value of χ^2 test for association between an *HER2* FISH ratio < 2.0 and a lack of HER2 overexpression (ie, IHC0 and IHC1+).

‖*P* value of χ^2 test for association between an *HER2* FISH ratio ≥ 2.0, with either an average *HER2* gene copy number < 4.0, or ≥ 4.0 but < 6.00, and a lack of HER2 overexpression (IHC0 and IHC1+).

¶*P* value of χ^2 test for association between an *HER2* FISH ratio ≥ 2.,0 with an average HER2 gene copy number ≥ 6.0, and HER2 overexpression (IHC3+).

#*P* value of χ^2 test for association between an *HER2* FISH ratio ≥ 2.0 (without regard to average *HER2* gene copy number/tumor cell nucleus) and HER2 protein overexpression (IHC3+).

number of ≥ 4.0, had improved DFS and OS when treated with trastuzumab compared with those treated with conventional (AC-T) chemotherapy alone (n = 3,109; DFS: HR, 0.71; 95% CI, 0.60 to 0.83; $P < .0001$; and OS: HR, 0.69; 95% CI, 0.55 to 0.85; $P = .0006$; Tables 3 and 4).

ASCO-CAP group 2 (ISH positive), HER2-to-CEP17 ratio ≥ 2.0 and average HER2 copy number < 4.0. Among patients who were randomly assigned to BCIRG-006 trial of adjuvant trastuzumab whose breast cancers had an *HER2* FISH ratio of ≥ 2.0 but average *HER2* copy number of < 4.0/tumor cell, there was no apparent benefit from trastuzumab therapy, either in terms of DFS (n = 46; HR, 1.10; 95% CI, 0.31 to 3.89; $P = .886$) or OS (HR, 3.15; 95% CI, 0.35 to 28.63; $P = .284$; Tables 3 and 4).

ASCO-CAP group 3 (ISH positive), HER2-to-CEP17 ratio < 2.0 and average HER2 copy number ≥ 6.0. Overall, patients with breast cancer in this FISH group who were accrued to BCIRG-005 had a worse DFS (HR, 2.50; $P = .0252$) and OS (HR, 2.35; $P = .0885$; Tables 3 and 4) than did the comparator group, group 5. However, during central laboratory FISH screening, patients whose breast cancers had *HER2* ratios of < 2.0 and average *HER2* copy numbers of ≥ 6.0/tumor cell were considered to consist of a minority of *HER2*-amplified breast cancers within a majority pool of *HER2*-nonamplified breast cancers. These cases were distinguished from one another by additional analyses[21,26,31,32] (Data Supplement). Most patients in this *HER2* FISH group were accrued to BCIRG-005 as not amplified, whereas few were accrued to BCIRG-006 through protocol amendment as amplified. This approach with separation into two subgroups is supported by HER2 IHC assay results (Data Supplement). Although we had divided group 3 breast cancers into two different subgroups—one eligible for BCIRG-005 with an average of 7.43 *HER2* genes/tumor cell, and the other eligible for BCIRG-006 with an average of 16.38 *HER2* genes/tumor cell—we considered the small numbers insufficient for definitive evaluation of this group in either BCIRG-005 or BCIRG-006.

ASCO-CAP group 4 (ISH equivocal), HER2-to-CEP17 ratio < 2.0 and average HER2 copy number ≥ 4.0 and < 6.0/tumor cell. Because patients with breast cancers that had a ratio of < 2.0 were considered *HER2* not amplified, these patients were accrued to the BCIRG-005 trial of sequential (AC-T) or concurrent (taxotere, adriamycin, and cyclophosphamide) chemotherapy.[19] Outcomes among these 176 patients did not differ significantly from outcomes in group 5 (DFS: HR, 0.923;

Table 3. Comparison of *HER2* Ratio and Average *HER2* Gene Copy Number and ASCO-CAP Groupings With Clinical Outcomes in BCIRG-005

HER2 FISH (*HER2*/CEP17) Ratio	*HER2* Copies per Cell	No. of Subjects	DFS, No. of Events	OS, No. of Events	DFS HR (95% CI) and *P* for Log-Rank Test*	OS HR (95% CI) and *P* for Log-Rank Test*	ASCO-CAP FISH Group
< 2.0	< 4.0	3,079	971	606	1.0 (reference)	1.0 (reference)	Group 5
	4.01-6.0	176	51	30	0.923 (0.697 to 1.224) *P* = .5795	0.878 (0.609 to 1.267) *P* = .4872	Group 4
	≥ 6	11	6	4	2.502 (1.121 to 5.583) *P* = .0252	2.351 (0.879 to 6.284) *P* = .0885	Group 3

NOTE. The hazard ratios are for each ASCO group compared with ASCO Group 5 taken as the reference. There were too few patients accrued to BCIRG-005 with a *HER2* FISH ratio ≥ 2.0 for analysis of DFS or OS.
Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; DFS, disease-free survival; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; OS, overall survival.
*Group 5 (reference) compared with each other group in BCIRG-005 (*HER2* not amplified breast cancers).

95% CI, 0.70 to 1.22; $P = 0.58$; and OS: HR, 0.88; 95% CI, 0.61 to 1.27; $P = 0.49$; Tables 3 and 4).

ASCO-CAP group 5 (ISH negative), HER2-to-CEP17 ratio < 2.0 and average HER2 copy number < 4.0/tumor cell. *HER2* status by FISH for these patients with breast cancer was considered *HER2* not amplified or ISH negative and served as the baseline comparison group for DFS and OS in the BCIRG-005 trial.

DISCUSSION

The most recent ASCO-CAP guidelines have again redefined *HER2* gene amplification as determined by ISH in a fashion that is different from prior definitions, particularly the FDA-approved package inserts for *HER2* FISH companion diagnostic assays,[33,34] which includes criteria used for BCIRG/TRIO clinical trials,[4,19,21,22,27] as well as prior 2007 ASCO-CAP guidelines.[15,16] Originally, *HER2* gene amplification was assessed by Southern blot using hybridization of a radiolabeled *HER2* gene probe compared with hybridization of a probe for a control gene, for example, arginase (*ARG1*),[28] myeloperoxidase (*MPO*),[8,35] or *TP53*,[36] as an internal control for amplification. A ratio between *HER2* and control signals ≥ 2.0 was evaluated as amplification. Subsequently, gene amplification was assessed by FISH using either CEP17[27,37] or another gene on the same chromosome[32] as an internal control, again with a ratio of ≥ 2.0 being considered as evidence for *HER2* amplification. Therefore, similar strategies have been used over a 30-year period to assess breast cancers as either amplified or not amplified. These criteria were used for enrollment in all major trials of trastuzumab,[2-5] lapatinib,[13,14] and, more recently, pertuzumab[11] and trastuzumab emtansine,[12] which demonstrated a clinical benefit for HER2-targeted therapies.

ASCO-CAP guidelines changed the *HER2*-to-CEP17 ratio used for amplification from ≥ 2.0 to ≥ 2.2 in 2007,[15,16] then changed the ratio back to ≥ 2.0 in 2013[17] and 2014[18] with the addition of formalized categories using average *HER2* copy numbers per tumor cell. Because these new criteria for amplification by ISH are likely to select somewhat different patient populations for HER2-targeted therapies, we retrospectively re-evaluated these issues with breast cancers that had annotated long-term clinical outcomes from our clinical trials.

Because *HER2* amplification is accepted as directly associated with protein overexpression,[8,22,38] a worse DFS and OS in the absence of HER2-targeted therapy,[27,28] and with improved outcomes with HER2-targeted therapy,[2-5,13] we used these as criteria for assessment of each newly defined ASCO-CAP group (Table 5). In these analyses, most patients experienced no change in *HER2* amplification status as determined by FISH, as ASCO-CAP groups 1 and 5 represent the vast majority of patients (approximately 95%) and because the status as amplified (group 1) and not amplified (group 5) is not changed by the new guidelines (Table 5). Although we find only a small minority of patients (approximately

Table 4. Comparison of *HER2* Ratio and Average *HER2* Gene Copy Number and ASCO-CAP Groupings With Clinical Outcomes in BCIRG-006

HER2 FISH (*HER2*/CEP17) Ratio	*HER2* Copies per Cell	No. of Subjects	DFS Control, Events/No. of Subjects	DFS Trastzumab, No. of Events/ Subjects	DFS, HR (95% CI)*	DFS *P* for Log-Rank Test*	OS Control	OS Trastzumab	OS, HR (95% CI)*	OS *P* for Log-Rank Test*	ASCO-CAP FISH Group
≥ 2.0	< 4.0	46	4/18	6/28	1.10 (0.31 to 3.89)	.8860	2/18	4/28	3.15 (0.35 to 28.63)	.2839	Group 2
	≥ 4	3,109	251/1,031	391/2,078	0.71 (0.60 to 0.83)	< .0001	138/1,031	202/2,078	0.69 (0.55 to 0.85)	.0006	Group 1
Total		3,155									

NOTE. The HRs are for trastuzumab treatment arms compared with control chemotherapy-only arm. There were too few patients (n = 5) accrued to BCRIG-006 with a *HER2* FISH ratio < 2.0 and ≥ 6.0 average *HER2* gene copy number/tumor cell for analysis of the HR.
Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; DFS, disease-free survival; *HER2*, human epidermal growth factor receptor 2; HR, hazard ratio; OS, overall survival.
*Trastuzumab-containing treatment arms compared with control (chemotherapy alone) treatment arm.

Table 5. Comparison of FISH Groups, FDA Guidelines Status, and ASCO-CAP Guidelines Status, and Associations With Outcomes in BCIRG Clinical Trials

FISH									
Ratio	Average *HER2* per Tumor Cell	Group	Frequency, %*	FDA Status†	ASCO-CAP Guidelines	HER2 Protein Expression	Prognosis (BCIRG-005 trial)	Response to HER2-Targeted Therapy (BCIRG-006)	BCIRG/TRIO Study Conclusion
≥ 2.0	≥ 4.0	1	40.8	Amplified	ISH positive	HER2 overexpression ($P < .0001$; IHC3+)	Not included in trial	Significantly improved outcomes	*HER2* amplified
≥ 2.0	< 4.0	2	0.7	Amplified	ISH positive	HER2 low expression ($P < .0001$; IHC0/1+)	Not included in trial	No significant benefit	*HER2* not amplified
< 2.0	≥ 6.0	3	0.5	Not amplified	ISH positive	Combination of HER2 low and overexpression	Indeterminate mixed category	Indeterminate, mixed category	Mixed *HER2* not amplified and amplified, on the basis of expression
< 2.0	≥ 4.0, < 6.0	4	4.1	Not amplified	ISH equivocal	HER2 low expression ($P < .0001$; IHC0/1+)	Not associated with worse outcomes	Not included in trial	*HER2* not amplified
< 2.0	< 4.0	5	53.9	Not amplified	ISH negative	HER2 low expression ($P < .0001$; IHC0/1+)	Not associated with worse outcomes	Not included in trial	*HER2* not amplified

Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; FDA, US Food and Drug Administration; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization; TRIO, Translational Research in Oncology.

*Frequencies are based on screened population in Table 1.

†FDA HER2 status is based on the 1997 HER2 INFORM-HER assay approval[27] (Ventana Medical Systems) and the 2002 FDA package insert related to the HER2 PathVysion FISH assay[20] (Abbott Laboratories).

5%) are affected by the new ASCO-CAP guidelines changes, our findings contradict the designations of these guidelines for groups 2, 3, and 4. Groups 2 and 4, designated ISH positive and ISH equivocal, respectively, by the ASCO-CAP guidelines, seem to be *HER2* not amplified on the basis of associations with a lack of protein overexpression (groups 2 and 4), a lack of response to trastuzumab treatment (group 2), and similar prognosis as group 5 for patients (group 4) treated with chemotherapy alone (Table 5). Overall, we observe approximately 99.3% agreement with initial FDA-approved guidelines and 94.7% agreement with current ASCO-CAP guidelines (Table 5). The 4.6% differential is related to the only two groups, groups 3 and 4, introduced by ASCO-CAP that lead to different assessments of HER2 status compared with FDA criteria. Finally, our observations indicate group 2, which represents 0.7% of breast cancers, is misclassified by both the FDA and ASCO-CAP guidelines as amplified and ISH positive (Table 5).

Although cancers in group 3 are designated as ISH positive by ASCO-CAP guidelines, our results suggest that this group is a mixture of *HER2* not amplified and amplified breast cancers, with the majority being not amplified on the basis of criteria described previously.[21,31,32] These categorizations are supported by associations with HER2 expression (Data Supplement Table S1), as well as similar findings from a clinical consultation practice where breast cancers with high average *HER2* gene copy number per cell are associated with HER2 protein overexpression (IHC2+ and IHC3+), whereas those with lower average *HER2* copy number per cell are associated with low HER2 protein expression by IHC.[26] Nevertheless, we did not have a sufficient number of cases in these subgroups in the BCIRG trials to separately evaluate their association with clinical outcomes.

Because the *HER2*-not amplified BCIRG-005 trial completed accrual in February 2003 and the *HER2*-amplified BCIRG-006 trial continued accrual until March 2004 with local laboratory IHC prescreening for approximately 60% of breast cancers submitted to the central laboratories, the prevalence of *HER2* amplification in the screened population increased from 26% while both trials were in the accrual stage[21] to 40% when BCIRG-006 completed accrual a year later. Nevertheless, the distribution of cases in groups 2, 3, and 4 are similar to those in our consultation practice where the ASCO-CAP group 1, or *HER2* amplification, rate is 18%.[26]

There are now nearly three decades of accumulated experience and published data studying this alteration in human breast cancers. Although guidelines are helpful, diagnostic judgment and long-term outcome data remain important in the evaluation of testing criteria.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

AUTHOR CONTRIBUTIONS

Conception and design: Michael F. Press, Dennis J. Slamon
Financial support: Michael F. Press
Administrative support: Michael F. Press, Valerie Bee, Ivonne Villalobos, Mary-Ann Lindsay
Provision of study materials or patients: Michael F. Press, Guido Sauter, Wolfgang Eiermann, Nicholas Robert, Tadeusz Pienkowski, John Crown, Miguel Martin, Vicente Valero, John R. Mackey, Martina Mirlacher, Dennis J. Slamon
Collection and assembly of data: Michael F. Press, Guido Sauter, Wolfgang Eiermann, Nicholas Robert, Tadeusz Pienkowski, John Crown, Miguel Martin, Vicente Valero, John R. Mackey, Valerie Bee, Yanling Ma, Ivonne Villalobos, Martina Mirlacher, Mary-Ann Lindsay, Dennis J. Slamon
Data analysis and interpretation: Michael F. Press, Guido Sauter, Marc Buyse, Hélène Fourmanoir, Emmanuel Quinaux, Denice D. Tsao-Wei, Ivonne Villalobos, Anaamika Campeau, Dennis J. Slamon
Manuscript writing: All authors
Final approval of manuscript: All authors

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Affiliations

Michael F. Press, Denice D. Tsao-Wei, Yanling Ma, Ivonne Villalobos, and Anaamika Campeau, University of Southern California Norris Comprehensive Cancer Center; Dennis J. Slamon, Geffen School of Medicine at the University of California Los Angeles, Los Angeles, CA; Nicholas Robert, Virginia Cancer Specialists/US Oncology Research Network, Fairfax, VA; Vicente Valero, The University of Texas MD Anderson Cancer Center, Houston, TX; Guido Sauter and Martina Mirlacher, University of Hamburg, Hamburg; Wolfgang Eiermann, Frauenklinik vom Roten Kreuz, Munich, Germany; Marc Buyse, Hélène Fourmanoir, and Emmanuel Quinaux, International Drug Development Institute, Louvain-la-Neuve, Belgium; Tadeusz Pienkowski, Postgraduate Medical Education Center, Warsaw, Poland; John Crown, Irish Cooperative Onoclogy Research Group, St Vincent's University Hospital, Dublin, Ireland; Miguel Martin, Instituto de Investigación Sanitaria Gregorio Marañón, Universidad Complutense, Madrid, Spain; John R. Mackey, University of Alberta; Mary-Ann Lindsay, Cancer International Research Group/Translational Research in Oncology, Edmonton, Alberta, Canada; and Valerie Bee, Cancer International Research Group/Translational Research in Oncology, Paris, France.

Support

Supported in part by grants from the Breast Cancer Research Foundation (M.F.P.), California Breast Cancer Research Program (M.F.P.), Tower Cancer Research Foundation (Jessica M. Berman Senior Investigator Award), a gift from Dr. Richard Blach (M.F.P.), and Entertainment Industry Foundation (M.F.P. and D.J.S.) as well as an endowed chair, the Harold E. Lee Chair for Cancer Research (M.F.P.). The project was also supported in part by Grant No. P30CA014089 from the National Cancer Institute. Support for these trials was provided to the Breast Cancer International Research Group (BCIRG), now Translational Research in Oncology. The sponsor and major funder of the BCIRG-006 trial was Sanofi, with additional support provided by Genentech. Trastuzumab was provided by Genentech free of charge for study patients in the United States and was purchased by Sanofi for all study patients in other countries. Docetaxel was provided by Sanofi for all study patients. The BCIRG-005 trial was sponsored by Sanofi. The BCIRG-007 trial was sponsored by F. Hoffman-La Roche Ltd and Genentech.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

HER2 Gene Amplification Testing by Fluorescent In Situ Hybridization (FISH): Comparison of the ASCO-College of American Pathologists Guidelines With FISH Scores Used for Enrollment in Breast Cancer International Research Group Clinical Trials

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Michael F. Press
Honoraria: Biocartis, DAKO, Halozyme, Puma Biotechnology, Cepheid, Ventana Medical Systems
Consulting or Advisory Role: Biocartis, Halozyme, Puma Biotechnology, Cepheid, Ventana Medical Systems
Research Funding: Cepheid (Inst)

Guido Sauter
No relationship to disclose

Marc Buyse
Employment: International Drug Development Institute
Stock or Other Ownership: International Drug Development Institute

Hélène Fourmanoir
Employment: Business and Decision Life Sciences

Emmanuel Quinaux
No relationship to disclose

Denice D. Tsao-Wei
No relationship to disclose

Wolfgang Eiermann
Consulting or Advisory Role: Roche
Speakers' Bureau: Roche
Travel, Accommodations, Expenses: Roche

Nicholas Robert
Consulting or Advisory Role: New Century Health
Research Funding: Sideout Foundation
Other Relationship: Paradigm Dx

Tadeusz Pienkowski
Honoraria: Roche, AstraZeneca
Research Funding: Roche, Pfizer
Travel, Accommodations, Expenses: Roche, Novartis

John Crown
Honoraria: Eisai, Pfizer
Research Funding: Eisai (Inst)

Miguel Martin
Consulting or Advisory Role: Genentech, Novartis, Amgen, Pfizer, Eli Lilly
Research Funding: Novartis (Inst)

Vicente Valero
Consulting or Advisory Role: Genentech
Research Funding: Genentech (Inst)

John R. Mackey
Stock or Other Ownership: Pacylex Pharmaceuticals
Honoraria: Eli Lilly, Roche, Pfizer
Patents, Royalties, Other Intellectual Property: Pacylex Pharmaceuticals

Valerie Bee
No relationship to disclose

Yanling Ma
No relationship to disclose

Ivonne Villalobos
Honoraria: Biocartis (I), Halozyme (I), Puma Biotechnology (I), Cepheid (I), DAKO (I), Ventana Medical Systems (I)
Consulting or Advisory Role: Biocartis (I), DAKO (I), Halozyme (I), Puma Biotechnology (I), Cepheid (I), Ventana Medical Systems (I)
Research Funding: Novartis (Inst), Eli Lilly (Inst), Cepheid (Inst)

Anaamika Campeau
Employment: Kaiser Permanente (I), Aetna (I), Lakeside Medical Group (I)
Travel, Accommodations, Expenses: Aetna (I)

Martina Mirlacher
No relationship to disclose

Mary-Ann Lindsay
Research Funding: Translational Research in Oncology

Dennis J. Slamon
Leadership: Biomarin
Stock or Other Ownership: Pfizer
Honoraria: Novartis
Consulting or Advisory Role: Novartis, Eli Lilly
Research Funding: Pfizer, Novartis
Travel, Accommodations, Expenses: Biomarin, Pfizer, Novartis

Acknowledgment

We thank the patients who enrolled in the trials and gave their consent as well as the Translational Research in Oncology (previously known as Breast Cancer International Research Group) Investigators who recruited them at each of the clinical sites and staff at those sites who supported the trial: Angela Santiago (University of Southern California [USC] central laboratory), Roberta Guzman (USC central laboratory), Armen Gasparyan (USC central laboratory), Jian-Yuan Zhou, MD (USC central laboratory), Rooba Wardeh, MD (USC central laboratory), Yong-Tian (Brandon) Li, MD (USC central laboratory), Hedvika Novotny (Basel, Switzerland), Sandra Schneider (Basel, Switzerland), and Rosemarie Chaffard (Basel, Switzerland) for technical assistance; Sandra Swain, MD, chair of the data and safety monitoring committee; and all steering committee and translational research committee members.